# A strong strand displacement activity of thermostable DNA polymerase markedly improves the results of DNA amplification

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The sensitivity and robustness of various DNA detection and amplification techniques are to a large extent determined by the properties of the DNA polymerase used. We have compared the performance of conventional *Taq* and *Bst* DNA polymerases to a novel *Taq* DNA polymerase mutant (SD DNA polymerase), which has a strong strand displacement activity, in PCR (including amplification of GC-rich and complex secondary structure templates), long-range PCR (LR PCR), loop-mediated amplification (LAMP), and polymerase chain displacement reaction (PCDR). Our results demonstrate that the strand displacement activity of SD DNA polymerase, in combination with the robust polymerase activity, provides a notable improvement in the sensitivity and efficiency of all these methods.

Sequence-specific DNA amplification has many applications in molecular biology and medical diagnostics. At present, there are two main strategies for amplifying a defined DNA sequence: polymerase chain reaction (PCR) and isothermal amplification. PCR relies upon instrument-based thermal cycling to denature template DNA, anneal the primers, and extend the primers using a thermostable DNA polymerase (such as *Taq* polymerase) in order to exponentially increase the amount of DNA. Isothermal amplification may require an initial high temperature to denature the DNA template, but all other steps occur at the same temperature. A variety of isothermal amplification methods have been developed: strand displacement amplification (SDA) (1,2), rolling circle amplification (RCA) (3), cross priming amplification (CPA) (4), loop-mediated amplification (LAMP) (5), and other techniques.

LAMP is a widely used method for sequence-specific isothermal DNA amplification that is suitable for clinical diagnostics (6). A notable increase in sensitivity and efficiency of LAMP is achieved by heat pre-denaturation of the DNA template (7–10). Without the pre-denaturation step, the sensitivity of detection of *Mycobacterium tuberculosis* in clinical specimens is reduced by 200-fold (7,10). To date, only polymerases from thermophilic *Bacillus* species, such as *Bst* DNA polymerase and its derivatives, are used in LAMP (11). These DNA polymerases possess a strong strand displacement activity; however, they are not stable at temperatures greater then 70°C.

Enzymes used in PCR (e.g., *Taq* DNA polymerase) possess high thermostability and robust polymerase activity but do not exhibit a strong strand displacement activity and are therefore not suitable for isothermal amplification methods such as LAMP. At the same time, new methods of DNA amplification such as polymerase chain displacement reaction (PCDR) (12) may require a DNA polymerase that combines the high thermostability of

## METHOD SUMMARY

We present a novel *Taq* DNA polymerase mutant, SD DNA polymerase, which has a strong strand displacement activity, and demonstrate its use in PCR (including amplification of GC-rich and complex secondary structure templates), real-time PCR, long-range PCR (LR PCR), loop-mediated amplification (LAMP) and polymerase chain displacement reaction (PCDR).

*Taq*, and the strong strand displacement activity of *Bst*.

SD DNA polymerase (www.bioron.net) is a novel *Taq* DNA polymerase mutant with a strong strand displacement activity that is suitable for both PCR and isothermal amplification. It combines features such as high thermostability (up to 93°C–94°C), 5′-3′-polymerase activity, 5′-3′- strand displacement activity, and a lack of exonuclease activity. In the present work, we compare the performance of SD DNA polymerase with the performance of *Taq* and *Bst* DNA polymerases in PCR, PCDR, and LAMP.

## Materials and methods

### Enzymes and reagents

SD DNA polymerase, SD HotStart DNA polymerase, SD reaction buffer, and DNA markers were supplied by Bioron GmbH, Ludwigshafen, Germany (www.bioron.net). *Bst* DNA polymerase (large fragment), λ DNA, and human HeLa gDNA were obtained from New England Biolabs, Inc. (Ipswich, MA). Commercially available *Taq* DNA polymerases and reaction buffers were obtained from: Promega Corporation (Madison, WI; GoTaq Flexi buffer, *GoTaq*, and *GoTaq* HotStart DNA polymerase); Bioline Limited (London, UK; *MyTaq* polymerase and MyTaq buffer); and Evrogen JSC (Moscow, Russia; *rTaq* polymerase and Encyclo buffer).

dNTPs and a murine cDNA library were supplied by Evrogen JSC. Group B *Streptococcus* (GBS) control template DNA and GBS primers were obtained from Meridian Bioscience Inc., (Cincinnati, OH).

Primers and probes (except GBS primers) were synthesized by Syntol JSC (Moscow, Russia). A 135 bp artificial hairpin template was synthesized by Evrogen JSC. All sequences are provided in Supplementary Table S1.

### LAMP reaction

LAMP was performed with control template DNA and primers for GBS. Assay reactions (50 μl) contained: 40 U of DNA polymerase (SD or *Bst*); 1× GoTaq or SD reaction buffer; 3.5 mM $MgCl_2$; 0.5 mM dNTPs (each); 2 μl GBS control template DNA and GBS primers: F3T3 0.2 μM, B3 0.2 μM, FIP 0.8 μM, BIP 0.8 μM, FL 0.8 μM, BL 0.8 μM. The reactions were carried out at 63°C for 45 min, with or without initial preheating at 92°C for 2 min.

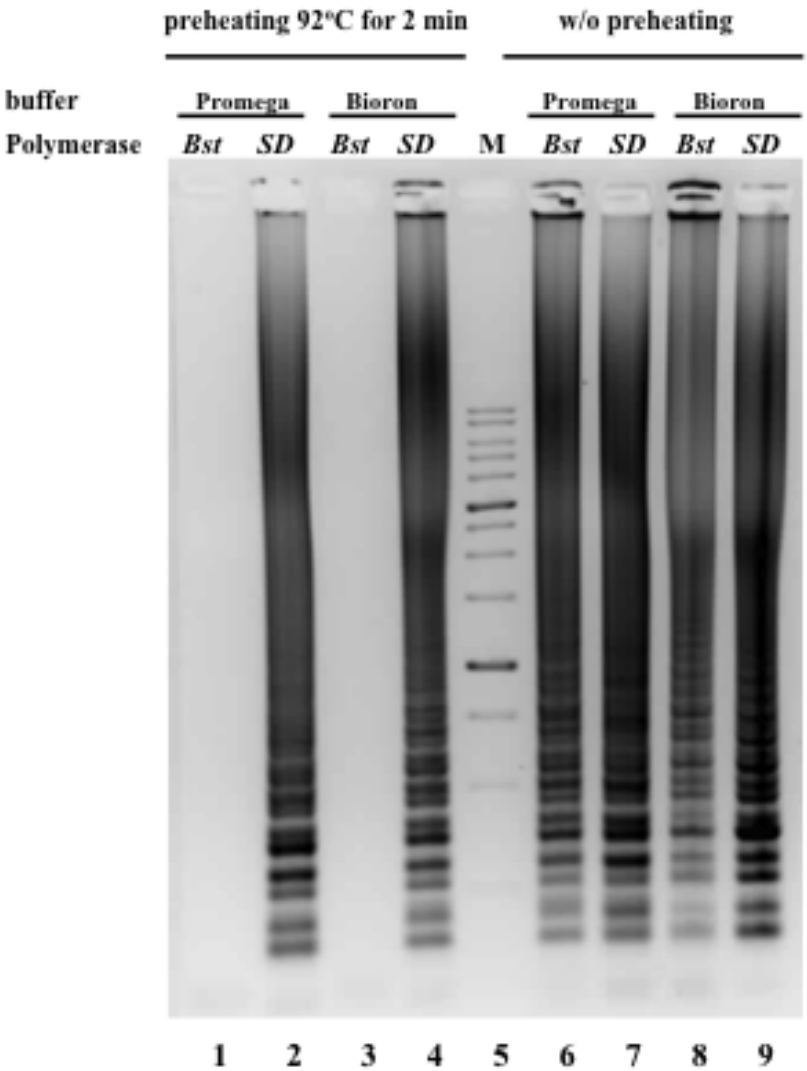


**Figure 1. LAMP DNA amplification with SD DNA polymerase and *Bst* (large fragment) DNA polymerase.** Reactions were carried out at 63°C with the indicated DNA polymerase (*Bst* or SD) and reaction buffers (Promega or Bioron), with (lanes 1—4) or without (lanes 6—9) the initial DNA denaturation step. M: 1 kb DNA ladder.

### PCR amplification

A 135 bp artificial hairpin DNA template was amplified with 2 U of SD or *GoTaq* DNA polymerase. Assay reactions (25 μl) contained: 1× GoTaq reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs (each), 0.1 ng template DNA, 0.2 μM primers H1 and H2 (each). Thermocycling conditions were: preheating 94°C for 1 min, followed by 15 cycles of 94°C (60 s), 64°C (20 s), 72°C (20 s).

A 1.3 kb fragment of the *Mycobacterium tuberculosis* genome was amplified with 1.25, 2.5, or 5 U of SD or *GoTaq* DNA polymerase. Assay reactions (25 μl) contained: 1× GoTaq reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs (each), 5 ng *Mycobacterium tuberculosis* gDNA as a template, 0.2 μM primers Mtu1 and Mtu2 (each). Thermocycling conditions were: preheating 92°C for 2 min, followed by 30 cycles of 92°C (20 s), 60°C (30 s), 68°C (2 min).

An 8 kb fragment of λ DNA was amplified with 2.5, 5, 10, or 15 U of SD polymerase, *GoTaq* polymerase (both

in GoTaq buffer), *MyTaq* polymerase in GoTaq buffer or MyTaq buffer; *rTaq* polymerase in GoTaq buffer or Encyclo buffer. Assay reactions (50 μl) contained: 5 ng λ DNA template, 0.25 mM dNTPs (each), 10 pmol (0.2 μM) of each primer (λ1 and λ2), 1× PCR buffer, and 3 mM $MgCl_2$ (GoTaq and MyTaq buffers) or 3.5 mM $MgCl_2$ (Encyclo buffer). Thermocycling conditions were: preheating at 92°C for 2 min, followed by 25 cycles of 92°C (30 s), 60°C (30 s), and 68°C (2 min 40 s; 20 s/kb).

Long-distance amplification of a 17.5 kb fragment of the human β-globin gene was performed with 2.5 U of SD polymerase for 35 cycles: 92°C (25 s), 66.5°C (1 min), 69°C (9 min); initial preheating 45 s at 92°C. Assay reactions (25 μl) contained: 100 ng of human gDNA (New England Biolabs) as template, 0.2 mM dNTPs (each), 5 pmol (0.2 μM) of each primer (HG1 and HG2), 1× SD buffer (Bioron), and 2.75 mM $MgCl_2$.

## PCDR and PCR amplification

A murine cDNA library was used as a template, and primers F1, F2, F3, R3, R2, and R1 (Supplementary Table SS) were designed to specifically amplify murine G3PDH cDNA. The relative position of the primers is shown in Figure 3A, the sequence of the amplified DNA, with the positions of the primers indicated, is given in Supplementary Figure S1. Assay reactions (50 μl) contained: 1× GoTaq buffer; 3 mM $MgCl_2$; 0.375 mM dNTPs (each); 20 pmol (0.4 μM) inner primers F3 and R3 (each) and 10 pmol (0.2 μM) outer primers F1 and R1; 0.05 ng of the murine cDNA library as a template. PCR assays contained two primers (F3, R3), and PCDR assays contained four primers (F1, F3, R1, R3). The reactions were carried out with 5, 10, 20, or 40 U of SD polymerase, or with 5 or 10 U of *GoTaq* polymerase. Thermocycling conditions were: preheating 92°C (1 min 30 s), followed by 20 cycles of 92°C (30 s) and 65°C (1 min).

## qPCDR and qPCR amplification

Real-time amplifications of murine G3PDH cDNA sequence were carried out with AmpliFluor primer AF3 (Syntol JSC) (Supplementary Table S1). The AmpliFluor primer is similar to inner primer F3 but includes a hairpin structure with a quencher (BHQ2) and a fluorescent reporter (HEX) at the 5′ end. A quantification cycle (Cq) is determined for each well with Bio-Rad (Hercules, CA) CFX Manager 3.0 by regression analysis. This method is based on the fit of Richards' equation to real-time PCR data by nonlinear regression in order to obtain the best fit estimators of reaction parameters (13). The efficiency of each reaction variant was estimated by standard curve (dilution series of murine cDNA library from 10 to 0.001 pg per reaction). The log of each concentration in the dilution series (x-axis) was plotted against the Cq value for that concentration (y-axis). Then efficiency was determined by the following equation:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

Assay reactions (25 μl) contained: 5 U of SD HotStart or *GoTaq* HotStart DNA polymerase; 1× GoTaq buffer for *GoTaq* polymerase or 1× SD buffer for SD polymerase; 2.75 mM $MgCl_2$; 0.25 mM dNTPs (each); 0.2 μM inner primers AF3 and R3 (each), 0.1 μM outer primers F2 and R2 (each), and 0.05 μM outer primers F1 and R1 (each); 10, 1, 0.1, 0.01, or 0.001 pg of the murine cDNA library as template.

PCR assays contained two primers, AF3 and R3. PCDR assays contained two inner primers (AF3, R3) and two (F2, R2) or four (F2, R2, F1, R1) outer primers.

Amplifications were carried out using a Bio-Rad CFX96 PCR machine with the following protocol: initial preheating 92°C 2 min, followed by 45 cycles of 92°C (15 s), 66°C (40 s).

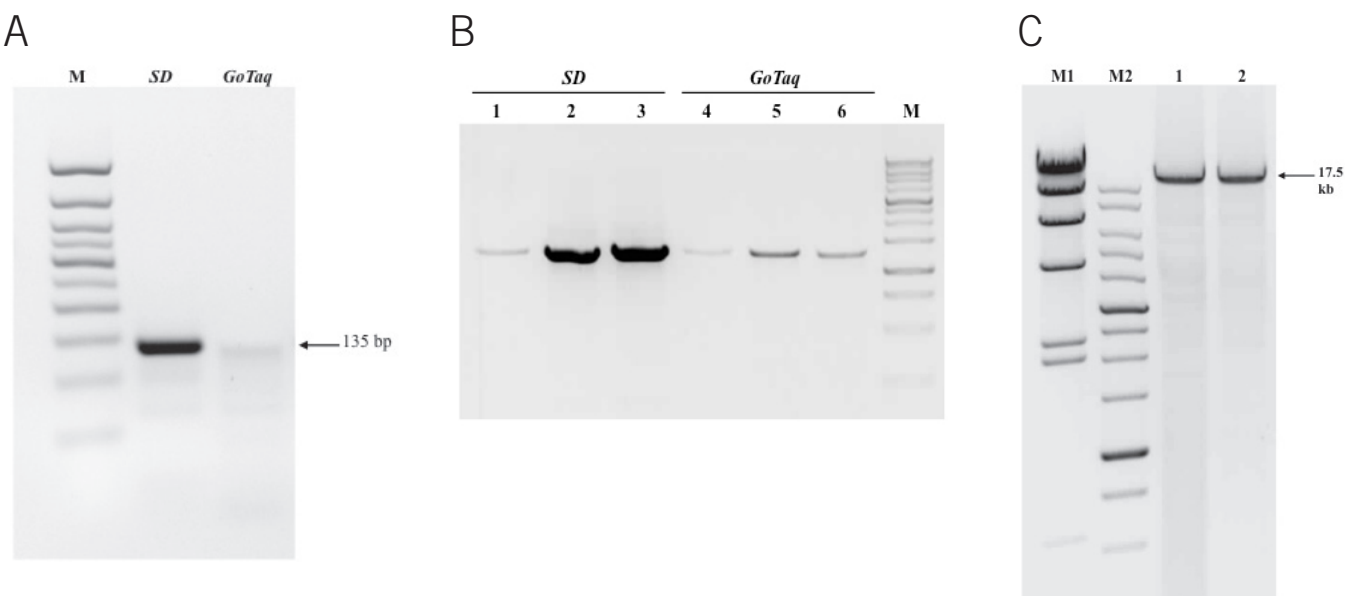


**Figure 2. PCR amplification with SD and *Taq* DNA polymerases.** (A) PCR amplification of a 135 bp artificial DNA template with a hairpin structure was carried out with the indicated SD and *GoTaq* DNA polymerases for 15 cycles. M: 50 bp DNA ladder. (B) PCR amplification of a GC-rich template. A 1.3 kb DNA fragment (64% GC) of the *Mycobacterium tuberculosis* genome was amplified with 1.25, 2.5, and 5 U of SD polymerase (lanes 1–3) and *GoTaq* polymerase (lanes 4–6), for 30 cycles. M: 1 kb DNA ladder. (C) Long-distance PCR with SD DNA polymerase. Amplification of a 17.5 kb fragment of the human β-globin gene was carried out with SD polymerase (lanes 1 and 2) for 35 cycles. M1: l/*Hin*dIII DNA marker; M2: 1 kb DNA ladder.

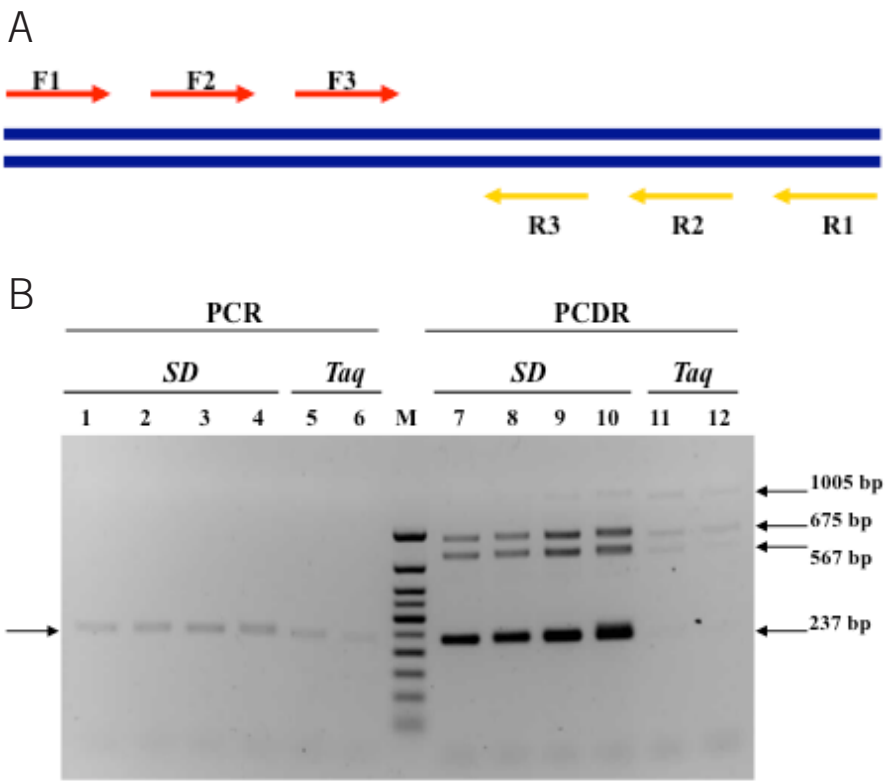


**Figure 3. SD DNA polymerase versus *Taq* DNA polymerase in polymerase chain displacement reaction (PCDR).** (A) Schematic drawing of the relative PCR and PCDR primer positions on murine G3PDH cDNA. (B) PCR (lanes 1–6) and PCDR (lanes 7–12) amplifications were carried out with 5, 10, 20, or 40 U of SD polymerase (lanes 1–4 and 7–10) or with 5 and 10 U of *GoTaq* polymerase (lanes 5, 6 and 11, 12). PCR assays contained two primers: F3 and R3. PCDR assays contained four primers: F1, F3, R3, and R1. Amplicon positions are indicated by arrows. M: 50 bp ladder.



## Results and discussion

### SD polymerase versus *Bst* polymerase in LAMP

We compared properties, such as strand displacement activity and thermostability, of *Bst* DNA polymerase (large fragment) to SD DNA polymerase in LAMP (Figure 1). Reactions were carried out at 63°C with or without an initial DNA pre-denaturation step at 92°C for 2 minutes. Without initial pre-denaturation, SD polymerase and *Bst* polymerase demonstrated very similar polymerase and strand displacement activities (lanes 6–9). However, with the pre-denaturation step, only SD polymerase was able to carry out the amplification (lanes 2 and 4), whereas *Bst* polymerase was completely inactivated at 92°C (lanes 1 and 3).

Thus, SD and *Bst* DNA polymerases had similar polymerase and strand displacement activities, but SD polymerase possessed much higher thermostability than *Bst* polymerase and therefore could be used in LAMP with the pre-denaturation step.

### SD polymerase versus *Taq* polymerase in PCR

We hypothesized that the strand displacement activity of SD polymerase could help to overcome problems amplifying templates that have extensive secondary structure and thus increase the efficiency of PCR. To test this, we synthesized a 135 bp artificial DNA template containing a hairpin structure with 30 complementary base pairs and compared SD and *Taq* DNA polymerase efficacy in PCR amplification. Only SD polymerase was able to provide an efficient amplification of the hairpin structure template (Figure 2A).

A 1.3 kb fragment of the *Mycobacterium tuberculosis* genome that has 64% GC content was amplified by SD and *Taq* DNA polymerases (Figure 2B). The SD polymerase provided a higher yield of the PCR product than *Taq* polymerase (lanes 1–3 versus lanes 4–6). The amplification results were not affected by the increase in the amount of *Taq* polymerase in the reaction (lanes 4–6).

Thus, we have shown that SD DNA polymerase was able to overcome problems with amplification of DNA templates with complex structures (GC-rich sequences or hairpins) much more efficiently than *Taq* polymerase. In these cases, the strand displacement activity of SD DNA polymerase becomes an advantage.

The quality and properties of *Taq* polymerase from different suppliers may sometimes differ, therefore we compared the efficiency of SD DNA polymerase to *Taq* polymerase from different sources by PCR amplification of an 8 kb λ DNA fragment. The results are shown in the Supplementary Figure S2. Independently of the source of *Taq* polymerase, the SD polymerase provided a markedly higher reaction efficiency.

### SD polymerase in long-range PCR

Amplification of long DNA fragments (over 8–10 kb) by means of long-range PCR (LR PCR) is a widely used technique in molecular biology. However, with an increase in the length of amplified sequence, the efficiency

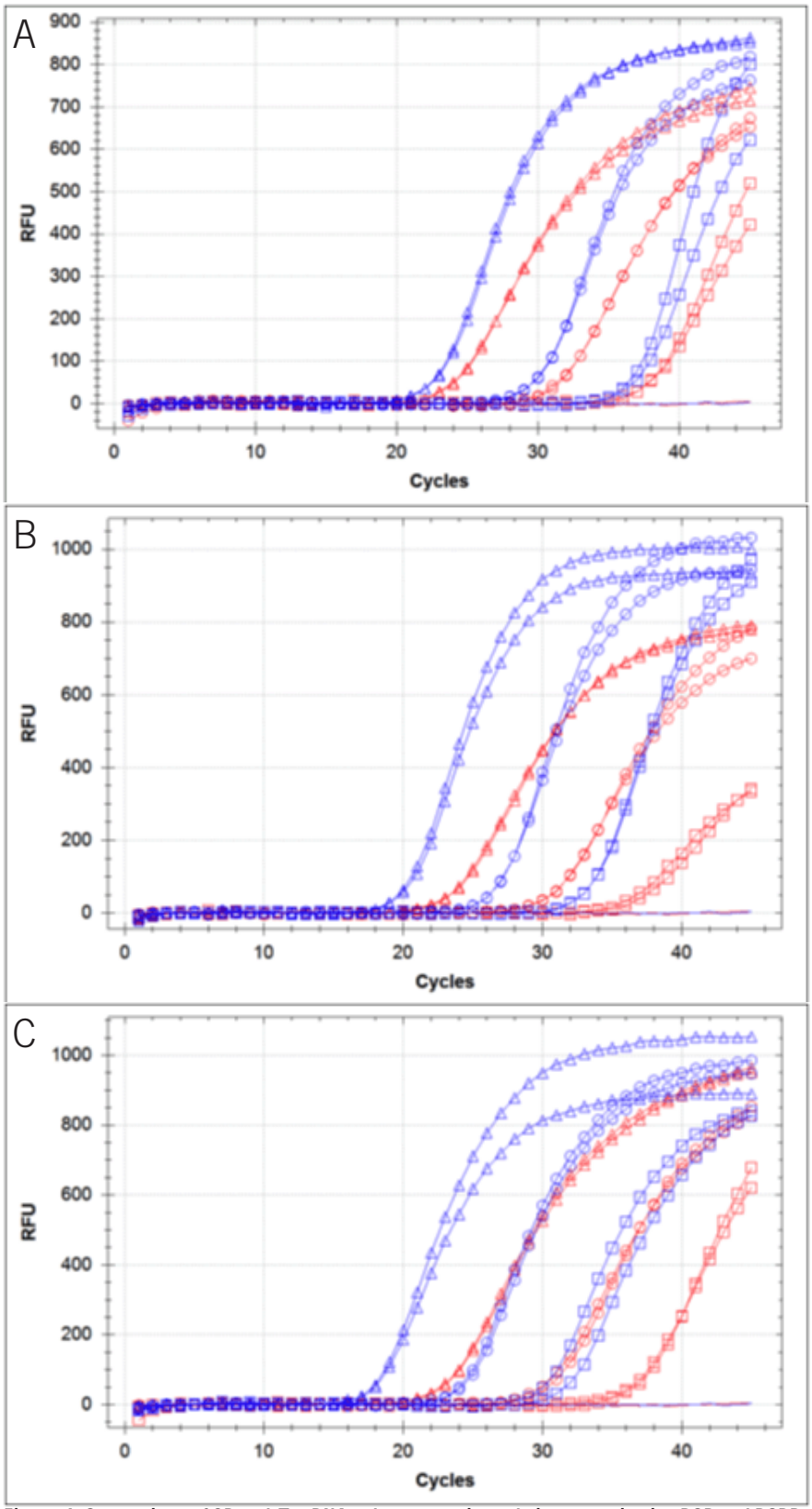


**Figure 4. Comparison of SD and *Taq* DNA polymerases in real-time quantitative PCR and PCDR.** Amplification of murine G3PDH cDNA sequence was carried out with 5 U of SD HotStart (blue curves) or *GoTaq* HotStart (red curves) DNA polymerase. Reaction assays contained the following amounts of cDNA library: 10 pg (triangles), 0.1 pg (circles), 0.001 pg (squares) and no template control reactions. (A) PCR amplification was carried out with two primers: R3 and AmpliFluor AF3. (B) Tetra-primer PCDR was carried out with four primers: F2, R2, R3, and AF3. (C) Hex-primer PCDR was carried out with six primers: F1, R1, F2, R2, R3, and AF3.

of the reaction decreases. Enzymes that are used in conventional PCR, such as *Taq* or *Tth* DNA polymerases, are unable to carry out LR PCR with high efficiency. The method that substantially increased the efficiency of LR PCR consisted of adding an enzyme possessing 3′→5′ exonuclease proofreading activity (e.g., *Pfu* DNA polymerase) to aid *Taq* or *Tth* DNA polymerases, which lack this activity (14,15). This method of carrying out LR PCR has been the main approach to date.

The strand displacement activity of SD polymerase markedly increased the efficiency of PCR (Figures 2A and B, Supplementary Figure S2). Therefore we have tested the suitability of this polymerase for carrying out LR PCR. A 17.5 kb DNA fragment from human gDNA was amplified in the presence of 2.5 U of SD polymerase for 35 cycles. As shown in Figure 2C, SD polymerase was able to mediate the fast (30 s/kb) and efficient long-range amplification. It should be emphasized that this result was obtained by using the SD polymerase only, without the addition of *Vent* or *Pfu* polymerases.

Thus, using a thermostable DNA polymerase with a strong strand displacement activity could be a novel alternative approach to achieve highly efficient LR PCR.

## SD polymerase versus *Taq* polymerase in PCDR

PCDR is a novel method of DNA sequence-specific amplification and initially described by Harris et al. (12). PCDR requires heat denaturation of dsDNA, like conventional PCR, and the strand displacement activity of DNA polymerase, like LAMP.

In PCDR, at least four primers are employed in the reaction—amplification is initiated together from the outer primers and the inner primers. By using a DNA polymerase with strand displacement activity, PCDR enables increased template amplification per cycle compared with standard two-primer PCR.

To test SD polymerase in PCDR, we used tetra- and hex-primer assays. PCDR with four primers should generate four fragments: one long, or common, fragment (I); two middle fragments (II, III); and one short fragment (IV). Calcu-

**Table 1. Comparison of quantitative PCR and PCDR with *Taq* and SD DNA polymerase.**

| Template dilution | Cq number | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Two primers | | | Four primers | | | Six primers | | |
| | SD | *Taq* | ΔCq | SD | *Taq* | ΔCq | SD | *Taq* | ΔCq |
| 1 | 22.07 ± 0.14 | 23.24 ± 0.21 | 1.17 | 19.61 ± 0.09 | 23.15 ± 0.06 | 3.54 | 17.58 ± 0.10 | 22.58 ± 0.17 | 5.00 |
| $1/10^1$ | 25.80 ± 0.02 | 27.00 ± 0.01 | 1.20 | 22.90 ± 0.01 | 26.63 ± 0.24 | 3.73 | 20.86 ± 0.06 | 26.30 ± 0.20 | 5.44 |
| $1/10^2$ | 29.14 ± 0.05 | 30.43 ± 0.02 | 1.29 | 26.08 ± 0.13 | 30.06 ± 0.19 | 3.98 | 23.88 ± 0.03 | 29.83 ± 0.30 | 5.95 |
| $1/10^3$ | 32.07 ± 0.03 | 33.80 ± 0.09 | 1.73 | 28.94 ± 0.33 | 33.26 ± 0.07 | 4.32 | 26.45 ± 0.15 | 33.02 ± 0.19 | 6.57 |
| $1/10^4$ | 35.92 ± 0.13 | 37.10 ± 0.30 | 1.18 | 32.51 ± 0.00 | 36.21 ± 0.34 | 3.70 | 30.08 ± 1.05 | 35.72 ± 0.62 | 5.64 |
| Efficiency | 96.9% | 94.9% | | 106.1% | 102.0% | | 112.3% | 101.0% | |
| $R^2$ | 0.998 | 0.999 | | 0.998 | 0.998 | | 0.992 | 0.994 | |

Amplifications of a murine G3PDH cDNA sequence were carried out with of SD HotStart or *GoTaq* HotStart DNA polymerase in 10-fold dilutions of a murine cDNA library (from 10 to 0.001 pg per reaction). Reactions contained two (in PCR), or four or six (in PCDR) primers including AmpliFluor direct primer AF3.

lation of the reaction kinetics of amplification of these fragments is as follows:

(I) $\mathbf{2^n}$

(II), (III) $\mathbf{n \times 2^{(n-1)}}$ or $\mathbf{(n \times 2^n):2}$

(IV) $\mathbf{(n^2 + 3n) \times 2^{(n-2)}}$ or $\mathbf{(n^2 + 3n) \times 2^n:4}$

(where **n** is the cycle number).

Thus the PCDR amplification of the short fragment (IV) outperforms standard (two primer) PCR amplification $\mathbf{(n^2 + 3n):4}$ times (where **n** is the cycle number).

PCDR with six primers should generate nine fragments, with the following reaction kinetic of the shortest fragment amplification:

$$\mathbf{[(n^2 + 3n) \times 2^{(n-2)}]^2 + (n \times 2^n)}$$

(where **n** is the cycle number).

The PCDR outperforms PCR amplification $\mathbf{(n^2 + 3n)^2 \times 2^{(n-4)} + n}$ or $\mathbf{(n^2 + 3n)^2 \times 2^n:16 + n}$ times.

In our PCDR experiments, we used a murine cDNA library as a template and primers for murine G3PDH cDNA. The relative positions of the primers are shown in Figure 3A. Standard PCR was carried out with 2 primers, F3 and R3, and the amplification generated only a single 237 bp fragment (Figure 3B, lanes 1–6). The PCDR was performed with four primers: inner (F3, R3) and outer (F1, R1). In these conditions 4 fragments were generated: 237 bp, 567 bp, 675 bp, and 1005 bp (Figure 3B, lanes 7–12). When *Taq* polymerase was used in the reactions, the levels of PCR and PCDR products were about the same (Figure 3B; lanes 5, 6, 11, 12). Increasing the amount of *Taq* polymerase over 10 U per reaction did not increase the efficiency of the reaction; furthermore, inhibition of the reaction was observed (data are not shown). In contrast to *Taq* polymerase, SD polymerase generated much higher product levels in the PCDR amplification (Figure 3B; lanes 7–10) compared with PCR (Figure 3B; lanes 1–4). These results are in good correlation with our calculations of PCDR kinetics and show that SD polymerase (unlike *Taq*) could successfully be used in PCDR.

### SD polymerase versus *Taq* polymerase in real-time qPCR and qPCDR

To determine if SD polymerase could improve the sensitivity and efficiency of quantitative assays, we compared the SD HotStart and *GoTaq* HotStart DNA polymerases in real-time quantitative amplification of the murine G3PDH cDNA sequence. SD DNA polymerase does not possess the 5′–3′ exonuclease activity; therefore, TaqMan probes for performing real-time reactions could not be used, and an AmpliFluor (AF3) direct primer was used instead. AF3 was similar to inner primer F3 but included a hairpin structure with a quencher (BHQ2) and a fluorescent reporter (HEX) at the 5′ end (Supplementary Table S1). qPCR assays contained two primers: R3 and AmpliFluor AF3; qPCDR assays contained four (F2, R2, R3, and AF3) or six (F1, R1, F2, R2, R3, and AF3) primers, including AmpliFluor AF3 (Figure 3A). Figure 4 and Table 1 show that with SD HotStart polymerase, the quantification cycle (Cq) values were reduced by one cycle in qPCR (Figure 4A), by four cycles in tetra-primer qPCDR (Figure 4B), and by six cycles in hex-primer qPCDR (Figure 4C). The sensitivity of the hex-primer qPCDR with SD HotStart polymerase was 100 times higher than the sensitivity of qPCR with *GoTaq* HotStart polymerase (Table 1). The sensitivity of qPCDR with *GoTaq* HotStart polymerase was not significantly improved compared with qPCR (Table 1).

Our data and the data described in (12) indicate that PCDR assays could provide an efficiency of over 100%, which means a greater than 2-fold increase in amplicon levels per amplification cycle (Table 1). As we have shown here, the real-time amplification with SD HotStart polymerase significantly improved the sensitivity and the efficiency in all of the qPCDR and qPCR assays.

SD DNA polymerase possesses both high thermostability and strong strand displacement activity. In this study, we have shown that the high thermostability of the enzyme allowed DNA amplifications with dsDNA denaturation. The strand displacement activity of SD polymerase helped to overcome amplification problems associated with complex secondary structures in templates, and in combination with the robust polymerase activity, provided a highly efficient PCR and LR PCR. SD polymerase did not require the addition of an enzyme with proofreading activity to efficiently perform the LR PCR. SD DNA polymerase also improved the sensitivity and the efficiency of PCDR and real-time qPCDR. Thus, the properties of SD DNA polymerase make it suitable for various methods of sequence-specific amplification, from conventional PCR to multi-primer PCDR and isothermal LAMP, and provide a notable improvement in the sensitivity and efficiency of all these methods.

## Author contributions

K.B.I.: conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, critical revision;

E.V.B.: acquisition of data, critical revision; A.F.F.: acquisition of data, critical revision; K.A.B.: acquisition of data, critical revision; T.V.K.: interpretation of data, drafting the article, critical revision; V.M.K.: conception of the study, analysis and interpretation of data, drafting the article, critical revision, general supervision.

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## Competing interests

The authors declare no competing interests.

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